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CYTOTOXIC EFFECTS OF CONJUGATED LINOLEIC ACIDS ON HUMAN COLON CANCER CELLS (HT-29)

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SUMMARY

Conjugated linoleic acids (CLAs) are fatty acids found naturally in milk and meat products derived from ruminants and have been reported as anticarcinogenic agent in *in vitro* and *in vivo* studies. This study was conducted to assess the cytotoxic effects of *cis*-9, *trans*-11 (*c*9,*t*11), *trans*-10, *cis*-12 (*t*10,*c*12) and mixed isomers of CLA on human colon cancer cells (HT-29). Cells were grown on RPMI 1640 media and treated with different concentrations of CLA isomers for 72 hours. The results were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. The viability of HT-29 cells was reduced significantly ($P < 0.05$) by all CLA isomers tested in a dose-dependent manner. The median inhibitory concentration (IC_{50}) value varied with type of CLA isomer. Mixed and *t*10,*c12* were significantly ($P < 0.05$) more potent than *c*9,*t*11 CLA isomer. It has been confirmed that *c*9, *t*11; *t*10,*c12* and mixed isomers of CLA have cytotoxic effect on HT-29 cancer cells. Further studies are required to clarify their mechanism of action and to use them in the treatment and/or prevention of colorectal cancer.

Keywords: Conjugated linoleic acids, colon cancer cells (HT-29), MTT cytotoxicity assay, cytotoxicity

INTRODUCTION

Colon or colorectal cancer is one of the most common forms of gastrointestinal cancer in the world today (ACS, 2005). More than 940,000 cases are annually reported worldwide and nearly 500,000 die from this disease each year (WHO, 2003). The National Cancer Registry of Malaysia report showed that colon cancer ranks the third most common cancer for both men and women, with a higher rank than gastric cancer in Malaysia (Goh *et al.*, 2005). Even though age, polyps, family history, ulcerative colitis and lifestyle (obesity, smoking, excessive alcohol consumption and sedentary life) are the risk factors (Fernandez *et al.*, 2004), the incidence of colorectal cancer is highly related to type of diet consumed (WHO, 2003). Some dietary components that contain high saturated fatty acids are associated with high risk whereas consumption of diet with low saturated fatty acids is related to low risk of developing colorectal cancer (Reddy, 2004; ACS, 2005). Thus, different types of fatty acids have different effects in the promotion or prevention of colorectal cancer (Reddy, 2004; Theodoratou *et al.*, 2007). Conjugated linoleic acids (CLAs) are polyunsaturated fatty acids and are reported to have anticarcinogenic effect in cell culture and animal model studies (MacDonald, 2000; Bhattacharya *et al.*, 2006). They are isomers of octadecadienoic (18:2) acid with a conjugated double bond system. Conjugated double bonds are located at carbon atoms 7 and 9, 8 and 10, 9 and 11, 10 and 12, or 11 and 13 with all possible *cis(c)* and *trans(t)* configurations (Bhattacharya *et al.*, 2006). Although various isomers are formed by a combination of these arrangements, *c*9,*t*11 isomer of CLA is the most abundant, making up to 90% of the total CLA in meat and milk products of ruminants (Bauman *et al.*, 1999; Bhattacharya *et al.*, 2006). The second isomer which is commonly encountered is *t*10,*c*12 CLA (Mir *et al.* 2004; Lorenzen *et al.* 2007).

Conjugated linoleic acids (CLAs) are found predominantly in ruminant food products such as meat and milk (Bhattachrya *et al.*, 2006) as a result of microbial fermentation in the rumen. The amount of CLA in ruminant meat and milk products has been reported to vary from 1.2-22.10 mg/g of fat (Tanaka, 2005; Schmid *et al.*, 2006).

There are published reports about antiproliferative effect of CLAs on human colon cancer cells (Cho *et al.*, 2003; Bozzo *et al.*, 2007; Huang *et al.*, 2007). Most published reports have used mixed isomer but the information available on separate effect of most common isomers such as *c*9,*t*11 and *t*10,*c*12 is still limited. At present, the most active isomer (s) of CLA has not been identified. Therefore, the objective of the present study

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was to assess the cytotoxic effects of commercially available CLA (*c*9,*t*11, *t*10,*c*12 and mixed) isomers on HT-29 colon cancer cells.

MATERIALS AND METHODS

Cell culturing

Human colon cancer cells (HT-29) were obtained from American type culture collection which was isolated from a primary colon cancer in a 44-year-old Caucasian female *(http://www.atcc.org)*. Cells were grown in RPMI 1640 media (Gibco® Invitrogen, Canada), that contain 100 U/mL penicillin (Gibco® Invitrogen, Canada), 100 μg/mL streptomycin (Gibco® Invitrogen, Canada) and 10% foetal bovine serum (Gibco® Invitrogen, Canada). Cells were routinely maintained and subcultured in 25 cm^2 plastic flasks at 37°C in a humidified CO_2 incubator (RS Biotech Laboratory Equipment Limited, UK) with 95% air and 5% CO_2.

MTT cytotoxicity assay

The cytotoxic effects of CLA isomers were assessed using MTT assay. The assay is based on the ability of viable cells to convert water soluble MTT reagent (tetrazolium salt) into a purple water insoluble formazan. It is possible to know the amount of viable cells in the plate by measuring spectrophotometrically the amount of formazan produced (Plumb, 2004; Huang *et al.*, 2007). Cells were seeded at a density of $1x10^4$ cells per well in a 96 well plate. After overnight incubation, cells were treated with *c*9,*t*11 (purity $\geq$96 %) and *t*10,c12 (purity $\geq$98 %) (Cayman Chemical Ltd, USA) and mixed (42% *c*9,*t*11, 44% *t*10,*c12*; about 10% *c*10,*c*12 and 5% of a mixture of others) (Sigma chemical Co., USA) CLA isomers, and 5-fluorouracil (Sigma chemical Co., USA) at concentrations of 5, 10, 20, 40 and 80 μg/mL. 5-Fluorouracil, the chemotherapeutic drug most often used to treat colorectal cancer (Casale *et al.*, 2004), was used as positive control. Serum free RPMI 1640 media was used to dilute and obtain the treatment concentrations. After 72 hours of incubation, 10 μL of MTT labelling reagent (Invitrogen™ Limited, UK) was added into each well. The plates were then incubated again for 4 hours. After this incubation period, excess MTT reagent was aspirated and 50 μL of dimethyl sulphoxide (Sigma chemical Co., USA) was added to each well and mixed thoroughly. The plate was then transferred to a microplate reader (Opsys MR™, Dynex Magellan Biosciences Company, USA) and absorbance was recorded at 540 nm. Each treatment at different concentrations and the untreated control were in triplicates, and the experiment was repeated at least three times.

Statistical analysis

Data were expressed as mean with their respective standard deviation and differences among treated groups were assessed using one way analysis of variance followed by Duncan's multiple range test and $P < 0.05$ was considered significant.

RESULTS

Figure 1 shows the percentage viability of HT-29 cells following treatment with different concentrations of CLA isomers and 5-flurouracil (positive control). The viability of cells was significantly (P<0.05) reduced by all CLA isomers used in a dose-dependent manner. Significant (P<0.05) reductions in cell viability were observed at a concentration as low as 20 μg/mL for *t*10,*c*12 and mixed, 40 μg/mL for *c*9,*t*11 isomers of CLA and 10 μg/mL for 5-flurouracil. The mean (n=3) median inhibitory concentrations (IC_{50}) for mixed, *t*10,*c*12; *c*9,*t*11 CLA isomers and 5-fluorouracil were 22.70 ±5.1, 36.98 ±18.6, 67.02±19.3 and 13.07 ±6.8 μg/mL, respectively. Comparison of these values indicated that mixed, *t*10,*c*12 and 5-fluorouracil were significantly (P < 0.05) more potent than *c*9,*t*11 CLA isomer. The IC_{50} values of mixed, *t*10,*c*12 and 5-fluorouracil were not significantly different.

DISCUSSION

Colorectal cancer is highly prevalent in developed countries. Nowadays, the incidence of the disease is increasing in the Asia-Pacific region due to dramatic socio-economic changes (Goh *et al.*, 2005). So it is becoming the most common forms of gastrointestinal cancer and causing huge loss of life each year throughout the world (WHO, 2003). Therefore, searching better treatment regimens or preventive means are ongoing quests. Diet contains a number of biologically active chemicals that may promote or inhibit the progress of colon cancer. Conjugated linoleic acids are dietary components that have anticancerogenic properties (MacDonald, 2000; Bhattacharya *et al.*, 2006).

In this study, CLA isomers reduced cells viability in a dose-dependent manner. The antiproliferative effect of CLA isomers on HT-29 colon cancer cell line has also been reported by Cho *et al.* (2003) who obtained a 55% reduction in number of viable cells after exposing cells at 20 μM for 4 days. Beppu *et al.* (2006) also reported inhibition of colon cancer cell proliferation following exposure to different isomers of CLA.

Individual CLA isomers have been shown to possess different biological activities (Bhattacharya *et al.*, 2006; Huang *et al.*, 2007). The IC_{50} value of *c*9,*t*11 CLA isomer

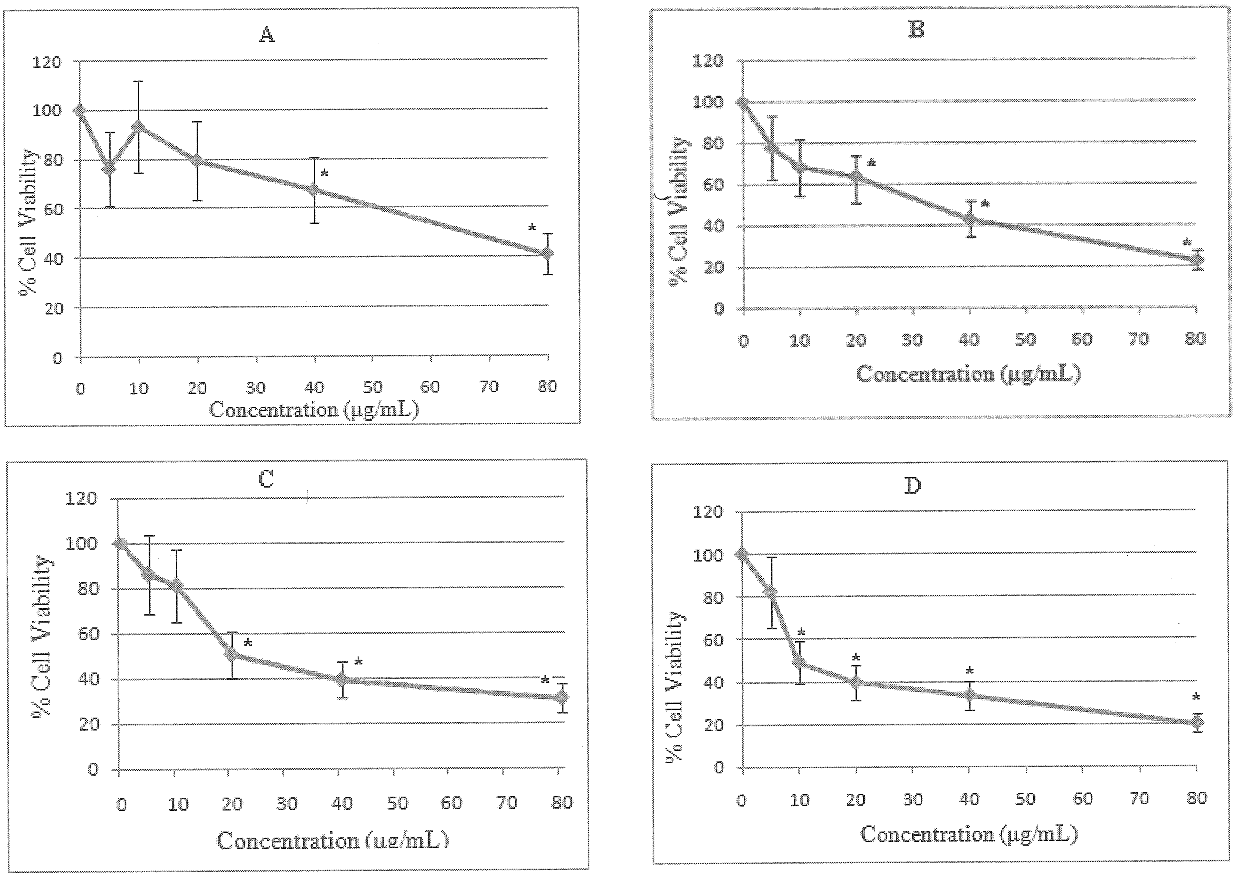

Figure 1: Viability of HT-29 cancer cells following treatment with CLA isomers and 5-flurouracil for 72 hours (Percent (%) cell viability was expressed as mean (n=3) percentage of untreated control values; *denotes significant (*P* < 0.05) difference from untreated control

A : Percent viability of cells treated with *c*9,*t*11 CLA isomer
B : Percent viability of cells treated with *t*10,*c*12 CLA isomer
C : Percent viability of cells treated with mixed CLA isomers
D : Percent viability of cells treated with 5-fluorouracil

was higher than other isomers tested. Isomeric variation on viability of HT-29 following treatment with CLA isomers was also reported by Beppu *et al.* (2006). According to the reports of these authors, *t*9,*t*11 showed strongest effect followed by *t*10,*c*12 and *c*9,*t*11 CLA isomers, respectively. The effect of mixed isomers may be related to one specific isomer or due to additive or synergistic effect in the mixed form.

Different suggestions have been made on the possible mechanism of action of CLA isomers to inhibit the viability of HT-29 cell lines. The inhibitory effect of *t*10,*c*12 CLA isomers on HT-29 cells was mediated through inhibition of insulin-like growth factor II secretion (Kim *et al.,* 2003). The inhibitory effect of CLA isomers on HT-29 cell was by inhibiting ErbB3 receptor signalling pathway (Cho *et al.,* 2003). Other studies relate the mechanism of action of CLA on HT-29 cells with increasing lipid peroxidation, alteration of cellular fatty acid composition and regulation of some gene expressions (Beppu *et al.,* 2006).

Antiproliferative effects of CLAs on other cancer cell lines have also been reported. Dose dependent reduction in MCF7 cancer cell viability as a result of CLA treatment was reported by Tanmahasamut *et al.* (2004) and Maggiora *et al.* (2004). Inhibitory effect of CLA isomers on human hepatoma cells (HepG2) cells was also reported by Igarashi and Miyazawa, (2001). In summary, CLAs are group of polyunsaturated fatty acids which inhibit cancer cell proliferation and viability. The present results warrant future research to use them in the regimen for fighting colorectal cancer.

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